Molecular and Epidemiological Assessment of Dengue Fever in Southern Sri Lanka in 2012

by

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October Sessions

Thesis submitted in partial fulfillment of
the requirements for the degree of
Master of Science
in the Global Health Institute
in the Graduate School
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ABSTRACT

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Abstract

Background. The mosquito-borne dengue viruses (DENV 1, 2, 3, and 4) have a rapidly expanding geographic range and have become endemic in over 100 countries with tropical and subtropical climates, including Sri Lanka. In Sri Lanka, dengue outbreaks have occurred primarily in Colombo, the capital, since the 1960s; however, recent reports suggest transmission throughout the island, with a second focus in the port city of Galle in the Southern Province.

Methods. To better assess the emergence of dengue among fever-causing agents in and around Galle, we collected epidemiological and clinical characteristics, as well as acute and convalescent sera, from febrile patients of 1 year of age or older without a defined source between June 1, 2012 and June 30, 2013 at the Teaching Hospital of Karapitiya. The distribution of serotypes was compared with similar data collected in 2007. We then performed whole genome sequencing on 40 dengue isolates from Colombo (n=20) and Galle (n=20) from 2012-13 to assess relatedness.

Results. Through serological testing for DENV IgM and IgG antibodies, virus isolation, and molecular testing, we confirmed acute dengue in 64.8% of the febrile population. Dengue serotype distribution in 2012 (DENV1 93.8%, DENV4 6.2%) differed from 2007, (DENV2 10.5%, DENV3 79%, and DENV4 10.5%). Phylogenetic analysis of whole genome sequence from representative samples from 2012-2013 demonstrated that most

DENV 1 strains (25 of 26 tested) from both Galle and Colombo belonged to genotype 1 and were closely related to strains previously reported in Colombo during the 2009-2010 dengue season. All DENV4 strains (n=10 from Galle and 10 from Colombo) belonged to genotype 1 and were most closely related to a strain from Thailand. These results support the movement of dengue virus strains from Colombo and Galle, but the timing and directionality of movement within Sri Lanka remain unknown.

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Acknowledgements

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My work on dengue in Sri Lanka was accomplished as part of a larger study of acute febrile illness (PIs Bodinayake, Nagahawatte, Reller and Woods). My time in Sri Lanka could not have been meaningful without the support and guidance from Dr. Ajith Nagahawatte for laboratory guidance and Dr. Champika Bodinayake for clinical assistance. I would like to also thank the molecular laboratory staff at the University of Ruhuna, Ms. Padmani, Mr. Dharmasiri, Mr. Aravinda and Ms. Dayalatha for their time through the training process as well as meeting my daily laboratory needs. ELISA set up would have been very difficult without the continued support and assistance from Dr. Gayani Tillekeratne, administrative guidance from Ms. Ruvini Kurukulasooriya and thoughtful inputs and emergency reagent deliveries by Dr. Dharshan de Silva of Genetech Research Institute, Colombo, Sri Lanka.

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To my family and friends around the world, this thesis is a product of your undying support and words of encouragement. Thank you for always being there and believing in me. Lastly, thank you to the Duke Global Health Institute and Duke University Center for International Studies for providing the financial assistance in making this proposal become a project.

Dedication

This thesis is dedicated to my mother for giving me the love and support as I follow my dreams.

1. Background

1.1 Global Dengue Burden

Dengue is the most rapidly spreading mosquito-borne viral disease. Due to the optimal environmental conditions of the tropical and subtropical regions of the world, roughly 3.6 billion people in these regions are at risk of contracting the disease, with an estimated 390 million new infections including 96 million symptomatic infections and 2 million dengue hemorrhagic fever (DHF) cases annually on a global scale (WHO, 2012; Bhatt et al., 2013; Beatty et al., 2008; Figure 1). Incidence of dengue has increased 30 fold in the past 50 years, posing a public health threat through human suffering, health system strain, and economic losses (WHO, 2012; Figure 2). Between 2000-2009, there were nearly 5 million cases of dengue and DHF cases worldwide.

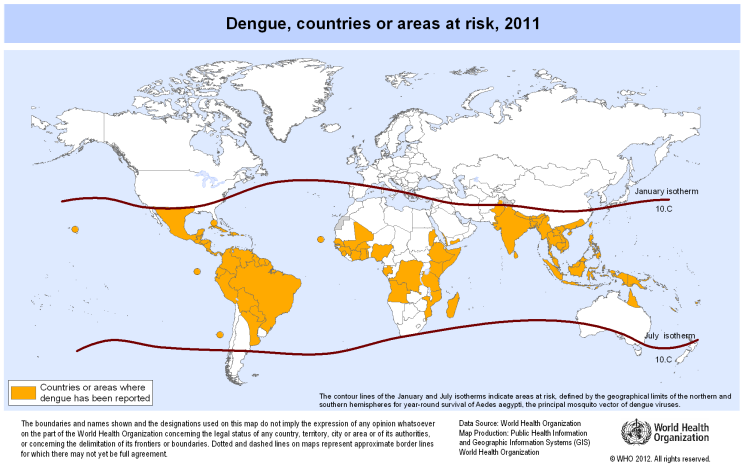


Figure 1: Countries and areas at risk of dengue as of 2011 (WHO, 2012)

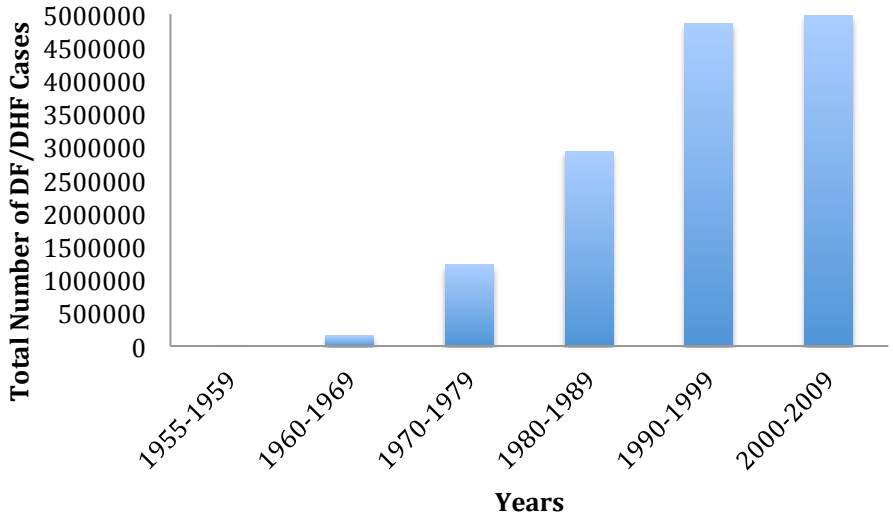


Figure 2: Total number of DF/DHF cases worldwide reported to the WHO from 1995 to 2009 (WHO DengueNet, 2014)

Although often asymptomatic, dengue fever (DF) is the most common symptomatic presentation of dengue virus (DENV) infection. Symptoms range from mild to incapacitating febrile illness accompanied by headache, bradycardia, abdominal tenderness, and muscle, joint and bone pain (Table 1; WHO, 2013). DHF and dengue shock syndrome (DSS) are more severe forms of DF where DHF is characterized by excessive plasma leakage selectively into pleural and peritoneal cavities accompanied with persistent vomiting, bleeding and breathing difficulty (WHO, 2013). DSS cases have all of the DHF symptoms present in addition to shock, or circulatory failure (Table 1). WHO has changed the working classification of dengue infection to be more sensitive to severe dengue (Figure 3; Hadinegoro, 2012). According to the new definitions,

approximately 2 million people are estimated to develop severe dengue infection annually (Bhatt et al., 2013). The case-fatality rate is estimated to be 2.5% across all forms of DENV infections, but ranges from 0.2% to 5% depending on severity of disease and access to appropriate, timely health care (WHO, 2009).

Table 1: WHO Definitions of Dengue Fever, Dengue Hemorrhagic Fever and Dengue Shock Syndrome (WHO, 1997)

Dengue Fever (DF)	• Acute febrile illness with two of the following: ○ Headache, retro-orbital pain, myalgia, arthralgia, rash, hemorrhagic manifestations, leukopenia • Supportive laboratory confirmation • Occurrence at same time and location of other confirmed DF cases
Dengue Hemorrhagic Fever (DHF)	All of the following: • Acute febrile illness • Hemorrhagic tendencies • Thrombocytopenia • Evidence of plasma leakage
Dengue Shock Syndrome (DSS)	All four criteria of DHF in addition to circulatory failure

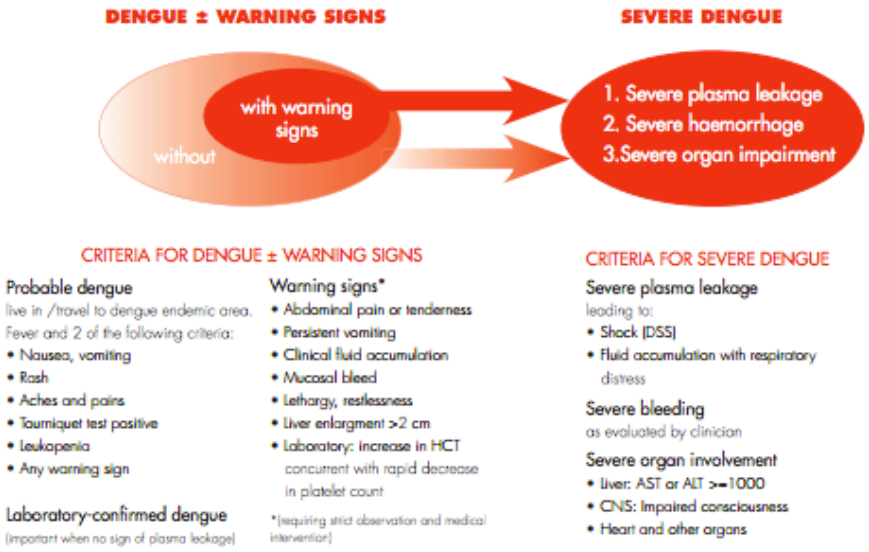


Figure 3: WHO Working Dengue Case Classification (WHO, 2009)

With dengue affecting a large portion of tropical developing nations, encompassing a half of the world's population, understanding the economic impacts of the disease is critical. Yet, very few studies have been conducted to assess these issues in developing nations. In 2004, an estimated annual cost of US$236 million was lost due to DF, DHF and DSS treatment, hospitalization, transportation and productive work loss in Southeast Asia (Shepard et al., 2004). Additionally with the impact of dengue outbreaks on tourism, the Southeast Asia region will lose an estimated US$2.36 billion during the next decade, or a comparable 264 disability-adjusted life years (DALYs) per million (population) per year for people in dengue risk areas (Cattand et al., 2006; Suaya et al., 2006). Although the economic burden of DF and DHF are not as great as other infectious diseases like malaria and HIV/AIDS, the existence of such a burden, especially considering the cyclical epidemic pattern of dengue outbreaks, warrants greater attention to decrease and prevent disruption in people's lives.

The social impacts of epidemics are difficult to quantify, however, are great enough to cause stress in health care systems and general public's perception of public health systems and the government. The overall ignorance to the importance of infectious disease surveillance and vector control in the latter half of the 20th century played a large role in the rise of emergence and re-emergence of numerous infectious diseases globally, especially in resource limited countries that do not have the financial or infrastructural capability to respond quickly to epidemics (Weiss and McMichael,

2004). In the case of dengue, the idea of living from epidemic to epidemic has been highlighted through the lack of individual involvement in vector control and ineffective governmental vector control programs (Gubler, 2002; Kendall et al., 1991). Varying levels of dengue awareness and prevention methods surveyed across many nations showed that commonalities lie in misconception of the disease and lack of knowledge and participation in household-level vector control (Itrat et al., 2008; Winch et al., 2002; Perez-Guerra et al., 2005). Although the actual social impacts are difficult to measure, such studies suggest the importance of public health efforts to educate the public on the disease and to emphasize individual involvement in vector control.

The origins of DENV are not clearly understood primarily due to the wide geospatial spread of the most recent common ancestors of the virus (Weaver and Vasilakis, 2009; Kuno et al., 1998). Phylogenetic and E-gene sequencing suggest that all endemic DENV serotypes arose from their sylvatic counterparts as early as 4000 years ago (Weaver and Vasilakis, 2009; Rico-Hesse, 1990; Wang et al., 2000; Gubler, 1997). The first epidemics of dengue were reported in Asia, Africa and North America by the late 18th century, but the overwhelming burden of dengue was not experienced until the post WWII era (Gubler, 2006).

In the mid 1950's, a program was initiated to control yellow fever through the eradication of the *Ae. aegypti* mosquito, the primary vector for both yellow fever virus (YFV) and DENV populations in the Americas using dichlorodiphenyltrichloroethane

(DDT). This effort largely reduced mosquito populations in the Americas and contributed to the low levels of dengue cases shortly after the program was initiated (Gubler, 2004). However, in the 40 years since the DDT program was halted due to environmental concerns, populations of *Ae. aegypti* mosquitoes and DENV have exploded (Figure 4) and the disease is now endemic in more than 100 countries across Africa, the Americas, the Western Pacific, the Eastern Mediterranean and south and Southeast Asia (WHO, 2013).

Figure 4: Geographic Distribution of DHF in the Americas pre-1981 and post-1981 (Gubler, 2011)

It is unlikely that the rapid spread of dengue and its vector is due to a single cause; poorly planned urbanization, global connectivity through trade and air travel are all thought to be important contributors. A large percentage of urbanization occurs in less developed nations where the annual urban population rate of change is 2.02%,

which is substantially larger than the 0.52% observed in more developed nations (UN, 2011). The *Aedes* mosquito is predominantly an urban mosquito with a preference for domestic and peridomestic breeding. Urban cities in tropical climates with rapidly increasing populations have proven to be particularly prime locations for feeding and breeding and are likely to increase the transmissibility of DENV (Gubler, 2011). Furthermore, urban areas in less developed countries also serve as temporary or permanent residences for many migratory workers who do travel back and forth to their rural homes, providing an avenue for disease transmission to occur from urban to rural areas. Traveling has also increased on an international scale. Roughly 900 million people traveled internationally in the past year (UN, 2013). Increased travel, as well as trade, potentially allows for wider spread of disease and vectors across countries and continents (Wilder-Smith and Gubler, 2008).

By the year 2020, the WHO hopes to reduce mortality and morbidity from dengue by at least 50% and 25% respectively, compared to the statistics from the year 2010 (WHO, 2012). To reach their goals, the WHO has proposed a multi-faceted approach that highlights the importance of global commitment and involvement in improving detection, management, and control of both disease and the vector. Of the 3.6 billion people currently living in regions of DENV risk, nearly 75% of the global population exposed to dengue is in the Asia-Pacific region (WHO, 2012). Increased tourism to the tropical areas with Southeast Asia alone reporting 248 million incoming

tourists for 2013 (WHO, 2012; Kester, 2014) highlights the difficulty in exposure management in a fast-moving global community. Various forms of prevention and control efforts ranging from removal of breeding sites to genetic modifications of the vector have shown varying levels of success (Wise de Valdez et al., 2010; Harris et al., 2011; Eisen and Beaty, 2008). Vector control is highly taxing on an economical and personal commitment level and its lack of success is reflected by the global burden of dengue (Alphey et al., 2010).

To date, there is neither specific therapeutic treatment nor preventative vaccine available for dengue. There are four serotypes of DENV: DENV-1, DENV-2, DENV-3, and DENV-4 where infection from one serotype provides lifelong serotype-specific immunity (Gubler, 1998) and only 2 months on average of cross protection from the other three serotypes (Snow et al., in press). Secondary infections of DENV are believed to be more severe due to an array of factors such as patient status and virus strain as well as the antibody-dependent enhancement (ADE) theory that illustrates the neutralizing limitations of heterotypic IgG antibodies (Thomas, 2008; Halstead, 1998).

Conventional treatment plans for dengue currently consist of analgesics and fluid replacement therapy since there is no pathogen specific treatment (CDC, 2014). Hence, developing an antiviral drug and/or vaccine that effectively covers all four serotypes is critical. Currently, there are six tetravalent vaccines that include live-attenuated candidate vaccines as well as inactivated, subunit and DNA candidate

vaccines (Gubler, 2011). The current leader in creating a dengue vaccine is Sanofi-Pasteur who has recently published results from the Phase IIb clinical studies in Thailand, where their live-attenuated, chimeric vaccine was found to be effective against three out of the four circulating serotypes in endemic populations. Phase III trials are currently underway in multiple countries where positive results may allow for vaccine rollout to begin as early as 2017 (Whitehead et al., 2007; Durbin et al., 2011; Sabchareon et al., 2012; Bernal, 2013).

1.2 Virus and Vector

DENV genome is made of positive sense single RNA strand that encodes a single polypeptide that is then post-translationally processed into ten mature viral proteins. Of the ten proteins, three – capsid, envelope, and pre-membrane – are structural proteins and the other seven – NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 – are non-structural proteins (Guzman et al., 2010).

DENV are mosquito-borne flaviviruses that are primarily transmitted to humans by *Aedes stegomyia* species mosquitoes. Although DENV can be carried by various species within the *Aedes* genus including *Ae. albopictus*, *Ae. polynesiensis* and *Ae. scutellaris*, *Ae. aegypti* mosquitoes have been shown to be the most efficient mosquito vectors for epidemic DENV primarily due to their domestic lifestyle and feeding behavior (Gubler, 1997). The female *Ae. aegypti* mosquitoes are known to be very

nervous feeders that commonly break up a single feeding event into multiple short events and often times from multiple sources (Gubler, 2006). This behavior allows for the rapid transmission of DENV to multiple people in a short period of time. Furthermore, *Ae. aegypti* mosquitoes prefer to lay their eggs in artificial containers containing water such as cisterns, tires, trash and flower pots – items that are commonly found in residential and urban areas (Gubler, 2006).

Once an individual is infected with DENV through a mosquito bite, the virus enters an incubation period lasting up to two weeks (average 4-7 days) before onset of illness. This is known as the intrinsic incubation period (IIP) (Siler et al., 1926; Sabin, 1952). With onset of fever, the individual enters the acute febrile viremic stage when the person can infect mosquitoes that feed on them. Once the mosquito ingests virus with infected blood, the virus begins replicating virions in the midgut epithelial cells (Ramirez et al., 2009). The virions then enter all areas of the hemocoel and ultimately infect the salivary glands before the mosquito becomes infective and develops the ability to transmit virus to another person. This is known as the extrinsic incubation period (EIP), which lasts 8-12 days, depending on ambient temperature (Gubler, 1998; Gubler, 2006).

1.3 Study Country: Sri Lanka

The Democratic Socialist Republic of Sri Lanka, or Sri Lanka as it is commonly known, is an island country of 65,610 km^2 located off of the coast of the Southeastern tip of India in the Indian Ocean (Figure 5). According to the World Bank, the total population is 20,328,000, making the country the 57th most populous in the world. Despite its classification as a lower middle-income country, Sri Lanka is beginning to show the vital characteristics characteristic of developed nations.

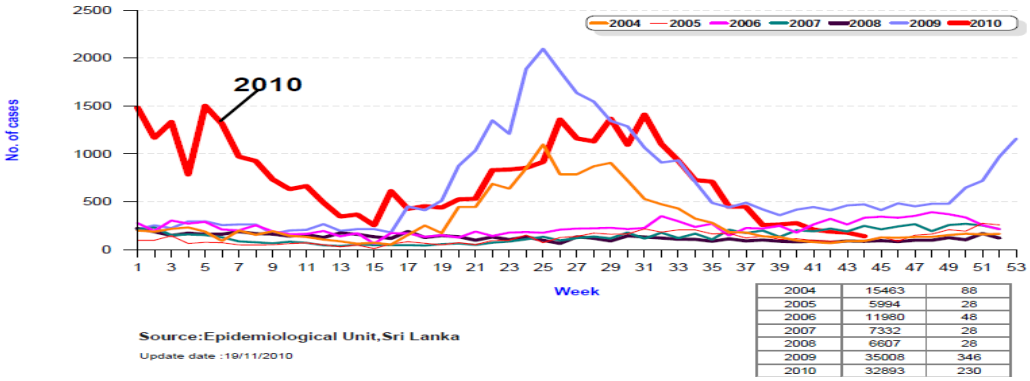
Figure 5: Map of Sri Lanka

1.3.1 Dengue Burden in Sri Lanka

Although the majority of dengue cases are not fatal, the cyclical epidemic breakouts since the early 2000s are of great concern. The first serologically confirmed dengue fever case was reported in 1962 with the first epidemic of dengue taking place from 1965-1968 (Vitarana et al., 1997). Prior to 1989, DHF was rare in the Indian subcontinent. Since 1989, especially in Sri Lanka, there has been a dramatic increase in the frequency of DHF and this region has been considered endemic ever since (Messer et al. 2002; Vitrana et al., 1997). Sri Lanka experienced its first epidemic with DHF in 1989 with 203 cases and 20 deaths and then again in 2004 with 15,463 cases and 88 deaths, with DENV 2 and 3 responsible for most cases (Wichmann et al., 2009; Kanakaratne et al., 2009; Epidemiology Unit of Sri Lanka). A significantly larger epidemic was observed in 2009 with 35,008 reported cases and 346 deaths, mainly caused by a new genotype of DENV 1 (Tissera et al., 2011).

Sri Lanka generally experiences two monsoon seasons annually, one spanning the months of May through July and the other spanning from late December through February. With the heavy rain and already humid tropical climate, monsoon seasons prove to be optimal times for mosquito breeding. Dengue cases have peaked during these monsoon seasons as highlighted from the number of cases reported to Colombo (Figure 7). The summer monsoon season has historically been associated with larger dengue outbreaks, however, in recent years, the winter monsoon season has also started

to show equally impactful outbreaks (Figure 8). In the most recent epidemic of 2012 there were 44,456 reported cases with 264 deaths (Epidemiological Unit of Sri Lanka). For the year 2013, 31,975 suspected dengue cases were reported with 51.63% of the cases coming from the Western province (Epidemiological Unit of Sri Lanka).



2004	15463	88
2005	5994	28
2006	11980	48
2007	7332	28
2008	6607	28
2009	35008	346
2010	32893	230

Figure 6: Number of cases reported to Colombo between 2004 and 2010 (Epidemiology Unit of Sri Lanka)

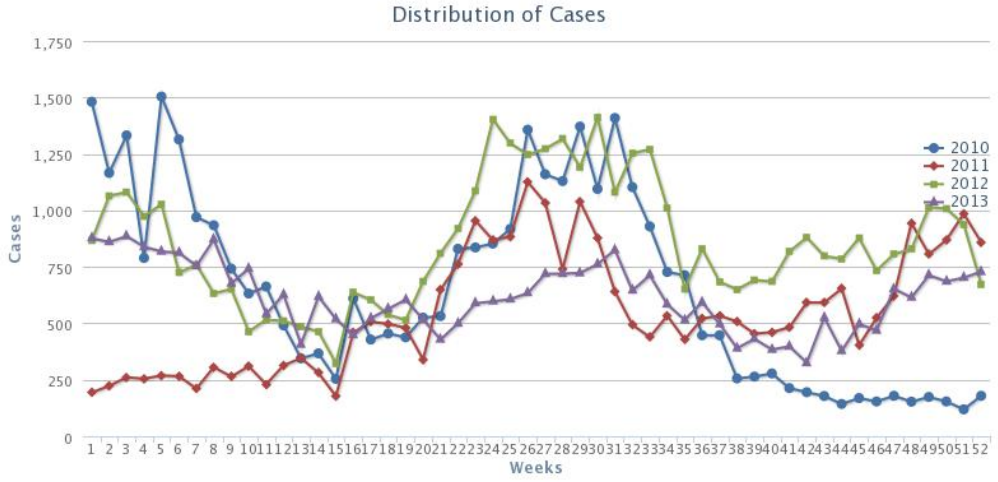


Figure 7: Number of cases reported to Colombo between 2010 and 2014

The rapid change in spatial representation of dengue cases within a 10-year span from 1996 to 2007 reveals the heavy concentration of cases in the Western Province, centering on the capital city, Colombo (Gubler 2004; Epidemiology Unit of Sri Lanka; World Health Organization, Figure 9). On average, close to 50% of all dengue cases occur largely in and around Colombo, in the central region by Kandy and along the southwestern shore. Incidence is higher during the annual monsoon seasons, May through July, which leads to higher humidity and optimal breeding sites for mosquitoes (Epidemiology Unit of Sri Lanka; Guha-Sapir et al., 2005).

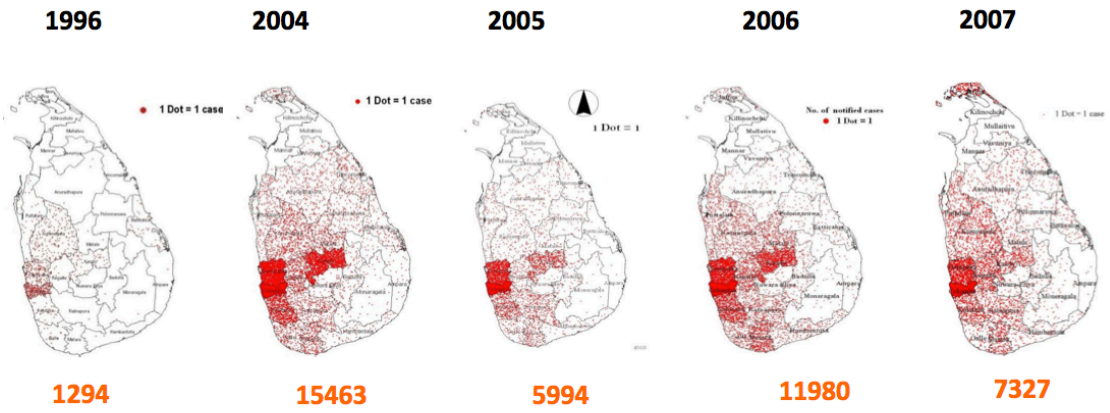


Figure 8: Spatial representation of annual dengue cases from 1996-2007 (Epidemiology Unit of Sri Lanka)

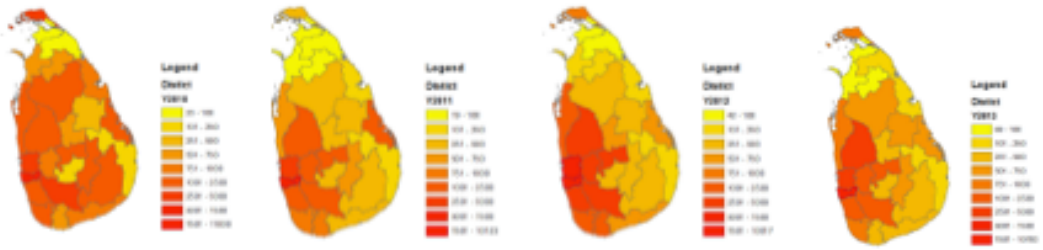


Figure 9: Spatial representation of annual DEN/DHF cases reported to Colombo from 2010-2013 (Epidemiology Unit of Sri Lanka)

Interchanging dominant serotypes have been observed with DENV2 detected in the 1980s followed by DENV3 for roughly two decades before leading to the current DENV1 epidemic (Kanakaratne et al., 2009; Messer et al., 2003). In the case of DENV-3, strain difference within the serotype has been linked to the epidemics in Sri Lanka, suggesting that circulating genotype differences in DENV-3 caused the recent spike in large outbreaks associated with severe disease from 1989-2004 (Messer et al., 2002; Messer et al., 2003). During the epidemic of 2009, a genotype 1 strain of DENV1 emerged and has been implicated as a significant contributor to increased circulation of DENV (Tissera et al., 2011). The re-emergence of DHF cases associated with strain variations in an endemic area like Sri Lanka illustrates the importance of genetic surveillance of dengue infections (Messer et al., 2002).

The age groups that have mostly been affected by dengue historically have been the youth. As of 1997, those under 15 years of age accounted for 65% of dengue cases

with the highest numbers of cases in the 5-9 years group (Vitarana et al., 1997). Since the early 2000s, however, not only has the number of total cases increased, but the proportion of dengue cases for those over 15 years of age surpassed the under 15 years of age (Epidemiology Unit of Sri Lanka). In a study from Colombo, the change in percentage of age groups affected is illustrated through the two groups that have been most affected: the under age-5 group and the 25-35 age group (Kanakaratne et al., 2009). This change in affected population is of great concern as the economic success that Sri Lanka has been experiencing is primarily due to the large work force created by the demographic bonus. The increased urbanization that Sri Lanka is facing with an influx of serologically naïve people into urban areas like Colombo suggest a reason behind this age shift. If the incidence of cases is high in the work force for a communicable disease, the susceptibility of contracting the disease increases, especially in densely populated urban areas. This poses a problem not only for the people who are immediately affected, but also to the population as a whole, in terms of disease spread.

Governmental support for dengue control has increased over the years with prevention programs specifically targeting the removal of breeding sites enforced on a national scale. Along with passive disease surveillance, vector control efforts have increased through a varying degree of solid waste disposal and container removal programs by district (Epidemiologic Unit, 2004). Public awareness of dengue has also increased through the help of media over the years, but there is always room for

improvement in boosting understanding and practicing of removal of breeding sites (Arunachalam et al., 2010; Gunasekara et al., 2012). Despite the rise in public awareness, prevention programs and improvements in medical knowledge, the number of dengue cases in Sri Lanka has continued to increase, making dengue illness a growing public health concern.

1.4 Study Objective and Aims

In order to better understand the current situation with dengue fever, as well as other febrile illness in southern Sri Lanka, a longitudinal, prospective study of febrile patients at the Teaching Hospital of Karapitiya (THK) was initiated in June 2012. We performed a sub-study in this larger surveillance study with a focus on dengue surveillance as Sri Lanka has been experiencing cyclical epidemics of dengue for several decades. Through serological and molecular assessments, we predicted the current dengue situation to differ in serotype distribution compared to our pilot study in 2007. We further sought to provide insight into virus strain dynamics by conducting full genome sequencing for selected samples from two study sites (Colombo and Galle) in Sri Lanka. We hypothesized that strains from Galle would be more similar to each other as compared to those from Colombo, which suggest strain divergence from Colombo and the possibility of local transmission.

2. Aim 1: Dengue Season 2012-2013

2.1 Study Population

Beginning in June 2012, febrile patients (T>38.0˚C) over 1 year of age were recruited from the Teaching Hospital Karapitiya in Galle, Sri Lanka as part of a collaborative program between Ruhuna University, Duke University, Duke-NUS Graduate Medical School, and Johns Hopkins University. Ethics review and approval was obtained from each of the participating institutions. The study was funded through a grant from the Office of Naval Research to the Program in Emerging Infectious Diseases, Duke-NUS Graduate Medical School, Singapore, and in part by a block grant from the Singapore Ministry of Health to Duke-NUS Graduate Medical School.

2.2 Sample Collection

Upon receiving informed consent from febrile patients, investigators completed a standardized instrument including the collection of demographic, clinical, and epidemiological data. Patients additionally returned for a follow up visit. Blood, urine and respiratory samples were collected at the acute visit and serum additionally at the convalescent visit.

2.3 Laboratory-Based Testing

Laboratory testing for both acute and convalescent samples were conducted at Duke-NUS Graduate Medical School as previously described (Gubler et al., 1984; Khan et al., 2013; Reller et al., 2012). Serological testing included dengue IgM and IgG antibody testing through ELISAs. Additional DENV isolation and molecular testing was done for serotypic analysis (Gubler, 1984; Johnson et al., 2005).

2.3.1 Dengue Serology

Dengue serology consisted of both the IgM and IgG antibody testing. Briefly for dengue IgM capture ELISA, inner 60 wells of a 96-well plate were coated overnight (4°C) with 75μL/well of goat anti-human IgM antibodies diluted at 1:2000 in a carbonate buffer (pH 9.6). Plates were blocked with 200 μL/well of blocking buffer (5% non-fat dairy milk (NFDM) in 1x phosphate buffered saline (PBS) and 0.005% Tween 20). Plates were washed 5x with 1xPBS before diluted serum (1:40) was loaded in triplicate and incubated (37°C for 1 hour). Unbound antibody was washed prior to overnight incubation with a mixture of DEN1-4 antigens and mouse anti-flavivirus IgG antibodies. Plates were washed before adding 50 μL/well of anti-flavivirus horseradish peroxidase (HRP) and incubated (37°C for 1 hour). After final washing, optical density (OD) was measured at 450nm upon final incubation with tetramethylbenzidine (TMB) substrates.

For dengue IgG ELISA, 96-well plates were coated overnight (4°C) with 100μL/well of dengue 2 purified antigen diluted to 117.6ng/mL in carbonate coating buffer (pH 9.6). Plates were washed 5x with 1xPBS and 0.05% Tween 20 before blocking with 100 μL/well with 1% NFDM and 1xPBS. After subsequent washing, 50μL of diluted serum and controls (1:100 in 0.5%NFDM in 1xPBS) were added as duplicates and triplicates, respectively, and incubated (37°C for 1 hour). Unbound antigens were washed and 25μL/well of goat anti-human IgG HRP antibody at 1:1000 in blocking buffer was added and plates were incubated. After final washing, OD was measured at 450nm upon final incubation with TMB substrates.

2.3.2 Dengue Virus Isolation

Isolation of DENV was conducted by inoculating C6/36 cell lines with acute sera from seroconverting patients. Briefly, 20μL of acute serum was diluted with 180μL of maintenance medium and added to C6/36 cells. Flasks were incubated at 33°C for 10 days with daily observation for cytopathic effects. Cells were tested for DENV with serotype-specific monoclonal antibodies using an indirect immunofluorescent antibody test. Supernatant were harvested and stored at -80°C.

2.3.3 Dengue PCR

Viral RNA for both Pan-Dengue and Pan-Flavi assays were extracted from acute sera using the QIAamp Viral RNA Mini kit (QIAGEN, Valencia, CA) following the manufacturer's protocols. The Pan-Dengue assay, a four-plex real-time RT-PCR (TaqMan) assay, was performed on all acute sera to confirm DENV serotype. Briefly, serotype-specific DENV primers and flurogenic probes were combined with viral RNA to determine DENV serotype. Acute sera that were negative on Pan-Dengue were tested on the Pan-Flavi assay, a single-plex TaqMan qPCR assay. Briefly, Pan-Flavi primers, Pan-Flavi probe was combined with viral RNA. PCR product was separated using a 1% TAE gel and sequenced. Sequences were blasted in PubMed to determine DENV serotype.

2.3.4 Laboratory Interpretation

We defined acute dengue as a case where virus isolation was positive, PCR was positive, and/or there was a seroconversion by IgG or IgM. Acute primary infection was defined as acute dengue with absence of IgG antibodies in acute sera. Those IgG or IgM positive in both the acute and convalescent sera were classified as acute secondary vs. past dengue. Seroconversion was defined through negative results on acute IgG and IgM ELISA and positive result on convalescent IgG and IgM ELISA. Seroprevalence was

defined for those with IgG positive results in the acute sera. For dengue IgM ELISA, one positive and one negative control were tested on each plate and were determined positive when mean OD value for the serum reacted on DENV antigen was at least twice the mean of the OD value for serum reacted on normal mouse brain antigen. For dengue IgG ELISA, two positive controls and one negative control were tested simultaneously per plate. Positive calls were made when mean OD values were higher than the mean+2SD value for the negative serum control per plate. For fluorescence assays, a four-tier scoring method was used where complete green fluorescence indicated strong positive and complete lack of green fluorescence indicated strong negative. Varying levels of fluorescence were subjectively scored compared to the positive and negative.

2.3.5 Full Genome Sequencing

Samples for full genome sequencing were selected to evenly cover a temporal range from May 2012 through March 2013 and were chosen based on a lower Ct value for the Pan-Dengue assay using the assumption that lower Ct values indicates higher viremia.

Procedures for full genome sequencing were based off of a method for sequencing the entire genome of all four DENV serotypes (Christenbury et al., 2010). DENV RNA was extracted primarily from acute sera but also from aliquoted culture supernatant using the QIAamp® Viral RNA Mini Kit (Qiagen™, Valencia, CA, USA) in accordance to the manufacturer's protocol and was stored at -80°C until further use.

cDNA synthesis was executed using SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen™, Carlsbad, CA, USA). 8 μL of viral RNA was combined with 1μL of 10 μM gene-specific synthetic oligonucleotide primer and 1 μL of 10mM dNTP mix in 0.2 mL RNAse-free microtubes. The final volume of 10 μL was incubated at 65°C for 5 minutes for denaturation in a Veriti 96Well Thermal Cycler from Applied Biosystems™ (Foster City, Ca, USA) before placing on ice for at least one minute. cDNA synthesis mix consisting of 2 μL of 10x RT Buffer, 4 μL of 25mM $MgCl_2$, 2 μL 0.1M DTT, 1 μL RNaseOUT™ and 1 μL of SuperScript™ III RT was mixed in a separate microtube and added to the denatured RNA mixture on ice. The RNA mixture with cDNA synthesis mix totaling a volume of 20 μL was incubated at 50°C for 50 minutes for cDNA synthesis and 85°C for 5 minutes for reaction termination on a Veriti 96 Well Thermal Cycler. 1 μL of RNase H was added to each microtube and incubated at 37°C for 20 minutes to remove RNA hybrid molecules. First-strand cDNA synthesis reactions were stored at -80°C until further use.

The DENV full genome for each serotype was divided into five overlapping amplicons. For each amplicon, 2 μL of cDNA was mixed with 5 μL of 10x PfuUltra II reaction buffer, 2.5 μL of 10 μM dNTP mix, 1 μL of 10 μM forward primer, 1 μL of 10 μM reverse primer, 1 μL of PfuUltra II fusion HS DNA polymerase and 38.5 μL of distilled water. PCR cycling parameters for the five amplicons can be found in Appendix A. PCR products were separated on a 1% agarose TAE gel and visualized under UV

light after staining with ethidium bromide (representative gel images in Appendix B). Bands of interest that were present without non-specific products were purified using a Qiagen QIAquick® PCR Purification Kit following manufacturer's protocol. Purified amplicons were sequenced by Sanger sequencing method (Sanger et al., 1977).

For amplicon creation and sequencing, published and unpublished primers were used (Christenbury et al., 2010). Primers developed during this study were created by identifying highly conserved regions within the region of interest after aligning multiple consensus full genome sequences for each serotype using Geneious® R6 (Biomatters Ltd. Auckland, NZ). A full list of sequencing primers can be found in Appendix C.

2.4 Analyses

Statistical analyses were performed on Stata IC 12.0 (StataCorp, College Station, TX). Categorical variables were compared by the χ^2 test or Fisher exact test. Continuous variables were analyzed by t-test or Kruskal-Wallis test.

For full genome sequencing, consensus sequences created through alignment with reference genomes were compared to other strains available in GenBank (National Center for Biotechnology Information) to generate maximum likelihood phylogenetic trees. Comparisons were conducted using the E-gene region only similar to what has been done previously in literature.

3. Results

3.1 2012-2013 Dengue Season

Enrollment began at the Teaching Hospital of Karapitiya (THK) at the end of June 2012 and has been ongoing since. Data presented here reflect the first year of study, from June 2012 through June 2013. Distribution of cases show a higher enrollment in July and August 2012 compared to the remainder of the first year (Figure 10). Selected descriptive statistics of the first year of samples with laboratory confirmation are shown in Table 2.

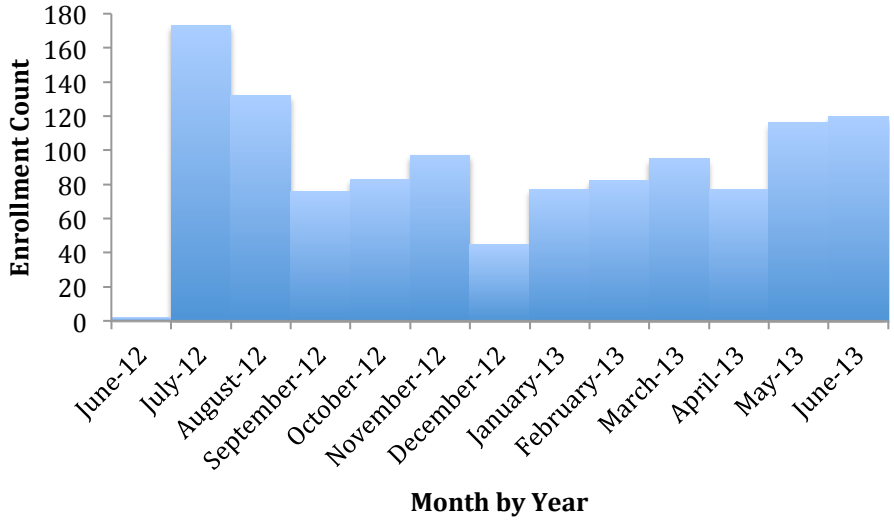


Figure 10: Enrollment graph from June 2012 - June 2013 (study started at the end of June 2012)

Table 2: Selected Demographic Variables for Laboratory Confirmed Dengue Cases*

Variable	Acute Dengue (n=270)	Secondary Acute or Past Dengue (n=123)	No Dengue (n=24)	p-value
Median Age, years (IQR)	31.2 (20.4 - 43.5)	42.5 (28.0 - 54.4)	11.8 (7.3 - 16.0)	<0.001
Median Days of Fever (IQR)	5 (3 - 6)	5 (4-7)	4 (2 - 5)	0.0055
Median Distance to THK, km (IQR)	30 (10-50)	20 (8-34)	13 (4-25)	0.001
Median Days to Conv. Visit (IQR)	24 (18-39)	26 (18-43)	26 (19-28)	0.383
Sex				0.974
Male	64.4	64.2	66.7	
Female	35.6	35.8	33.3	
Occupation				0.304
Housewife	16.8	18.6	0	
School	5	4.4	0	
Student	3.2	0.9	0	
Factory Laborer	11.4	13.3	25	
Agricultural Laborer	13.6	13.3	0	
Merchant	8.6	8.9	0	
Law Enforcement	4.6	3.5	0	
Construction Worker	2.3	4.4	25	
Other	25.5	20.4	0	
Retired	0.5	2.7	0	
Unemployed	7.73	8.9	50	
Education				0.064
Less than O/L	43.7	50.4	75	
O/L	27.8	18.7	20.8	
A/L	23.7	22.8	4.2	
Above A/L	4.4	7.3	0	
Travel				0.818
Provincial	12.6	8.1	4.2	
National	15.9	15.5	12.5	
None	70	75.6	83.3	

*Unless otherwise noted, all values are percentages

Analysis of the likelihood of a patient attending their convalescent visit did not differ based on sex, age, occupation or residence area. However, increased likelihood of attending their convalescent visit was found for children (P=0.026), those with less than O/L education (P=0.001) and those living less than 10km from THK (P=<0.001).

The days of fever prior to enrollment to the study were also analyzed with selected variables and found to not differ for sex, occupation, type of residence and education. However, shorter days of fever were observed for children (P=0.0001) and those who live less than 10km from THK (P=0.0098).

Distribution of the final clinical diagnosis upon discharge showed 24.2% of all diagnosis to be dengue and more than 50% of all diagnoses consisted of viral fevers (Table 3).

Table 3: Final Clinical Diagnosis Upon Hospital Discharge

Final Diagnosis	Frequency	Percentage
Dengue	284	24.2%
Other Viral Fevers	377	32.1%
Bacteremia	10	0.9%
Scrub Typhus	17	1.5%
Atypical Pneumonia	12	1.0%
Lower Repiratory Illness	129	11.0%
Meningitis/Encephalitis	15	1.3%
Leptospirosis	97	8.3%
Rickettsiae	3	0.3%
Upper Respiratory Illness	64	5.5%
Other	158	13.5%
missing	9	0.8%

620 samples of the 1175 enrolled in the first year have been tested further at Duke-NUS Graduate Medical School where dengue serotype was confirmed through Pan-Dengue and Pan-Flavi PCR assays. Of the 620 tested, 55.3% were found to be dengue positive. The breakdown of dengue serotypes showed 93.8% to be DENV1 and 6.2% to be DENV4 (Table 3). Days of fever prior to enrollment, with a median of 5 days, did not differ across serotypes (P=0.229).

Table 4: Dengue Serotype Observed in Dengue Season 2012-2013 (n=620)

Dengue Serotype	Frequency	Percentage
DENV1	321	51.9%
DENV4	21	3.4%
Negative	276	44.7%

Amongst the 1175 enrolled, paired sera – both acute and convalescent sera – were available for 417 at the time of analysis. Dengue serology was conducted on the paired sera and showed that acute dengue was present in 271 (64.8%) of the paired sera, of which 24.0% were primary infections. Probability of acute dengue did not differ by sex (P=1), but was higher for children (P<0.001) and urban areas (P=0.009). Overall seroprevalence for dengue was 79.9% where the largest proportion was observed in the 20-29 year age group with a seroprevalence of 30.3% (Figure 11). Seroprevalence statistically differed amongst the different age groups but not for other selected

variables including sex, education level, residence area, occupation and distance from THK.

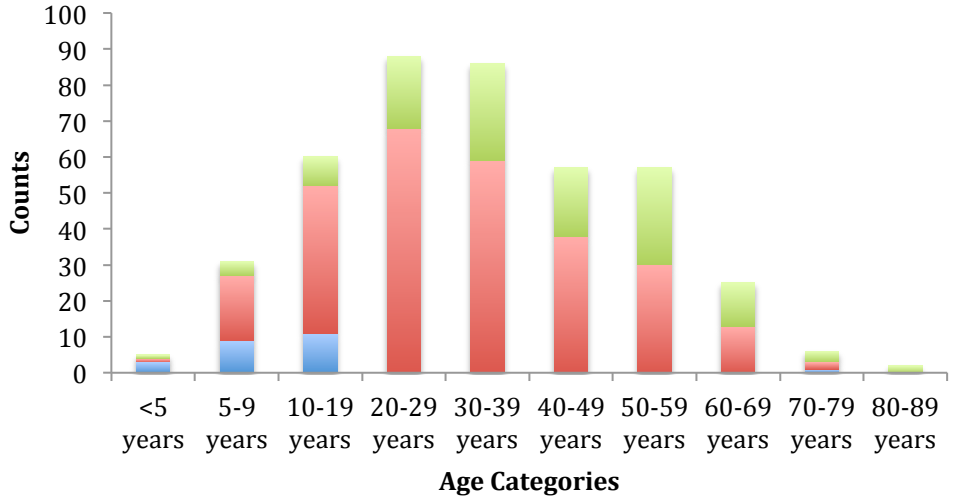


Figure 11: Distribution of dengue cases by age group (Blue - No dengue; Red - Acute dengue; Green - Past dengue)

3.2 Full Genome Sequencing

Phylogenetic analysis using full genome sequences were conducted for 40 viruses equally covering representative DENV1 and DENV4 serotypes. Half of the sequences came from the present Galle study and the other half from a separate study based in Colombo, Sri Lanka. No difference between sex and age of the patients was observed nor was there a statistical difference between days of fever prior to enrollment between samples collected from Galle and Colombo for each serotype.

A maximum likelihood mapping of the sequences was conduced for both DENV1 and DENV4 using the E-gene sequences. Two genotypes of DENV1, genotype 1

and genotype 5 were found amongst the 20 DENV1 samples (Figure 11). For DENV4 sequences, all viruses belonged to genotype 1 (Figure 12).

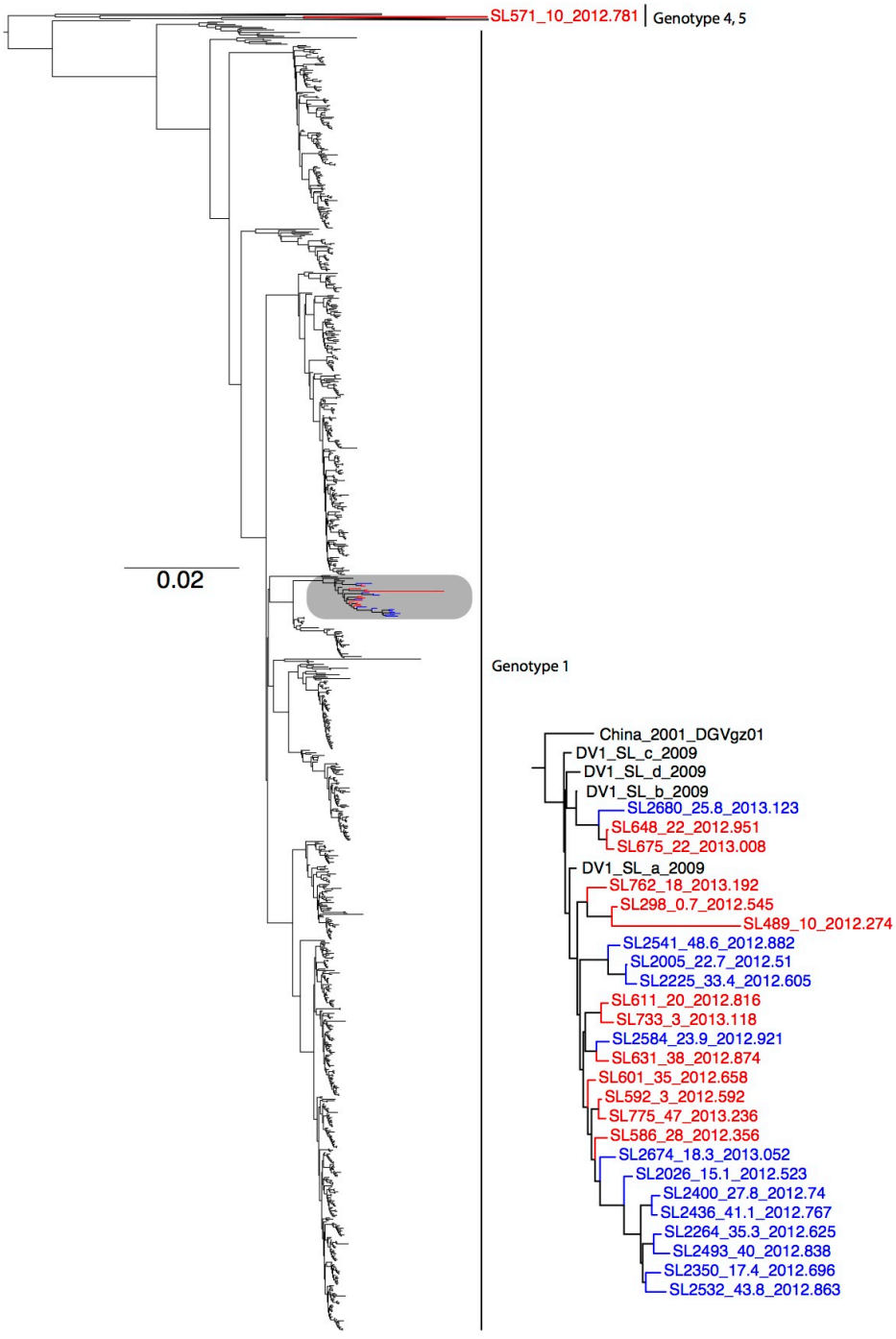


Figure 12: Phylogeny of DENV1 samples (red indicates samples from Colombo and blue indicates samples from Galle)

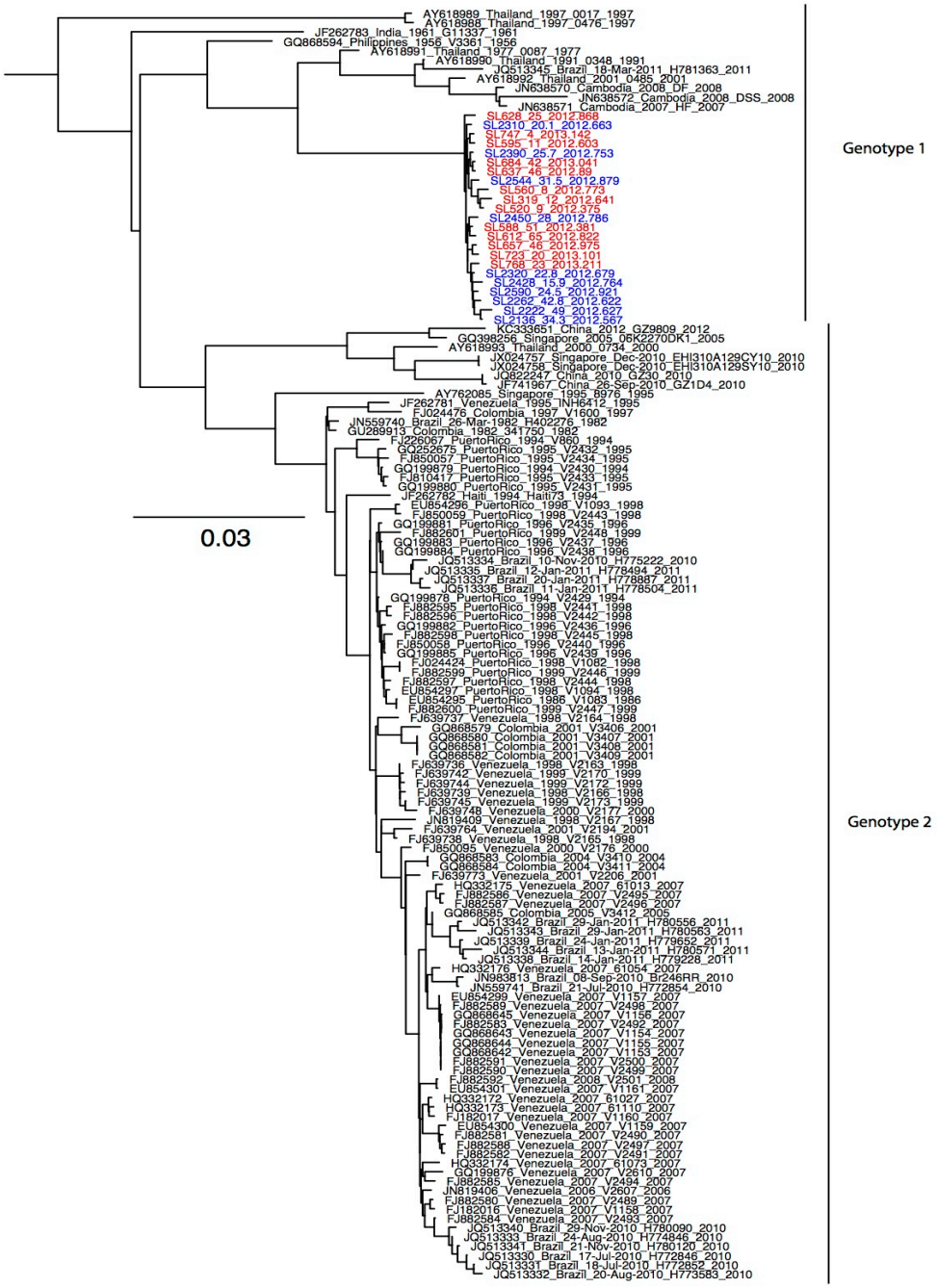


Figure 13: Phylogeny of DENV4 samples (red indicates samples from Colombo and blue indicates samples from Galle)

4. Discussion

4.1 Findings

Through the enrollment of febrile patients, we sought to characterize dengue in and around Galle, Sri Lanka. Laboratory testing of paired sera concluded that overall seroprevalence for dengue in this cohort is 79.9%, which not only increased with age similar to what was previously found in the same area, but also was a higher percentage than what was seen in 2007 (Reller, 2012). Serotype-specific analysis showed the predominance of DENV1 in the current study, which was first identified in Colombo in 2009, but was not present in Galle in 2007. Strain analysis for selected samples further confirmed relatedness of current DENV1 genotype 1 strains from both Colombo and Galle to that of DENV1 genotype 1 identified in Colombo in 2009. Phylogenetic analysis of the DENV1 and DENV4 samples also shows possible strain clustering for some of the Galle samples suggesting strain divergence and local transmission.

In the current study, compared to adults, children had a higher probability of seroconversion of 26.3% that decreased with increasing age, which is consistent with the fact that children have had less time for potential exposure to the virus as compared to adults, thereby limiting the probability that they would possess antibodies against a particular virus. Seroprevalence of dengue also reflected the limited amount of time children have had for potential exposure as 51.3% of children compared to 86.7% of

adults showed dengue seroprevalence. Children had an average of 4.3 days of fever prior to enrollment, which was found to be, on average, one day shorter than that of adults.

Among acute dengue cases, 25.2% of cases were seen in the 20-29 years age group followed by 21.9% in the 30-39 years age group. 20-29 years age group also saw the largest proportion of chikungunya cases at 24.6%. Our findings agree with the overall age shift from a median age of 15 years to a median age of 25 years that has been observed in Sri Lanka over the past decade (Sirisena, 2013; Tam, 2013). The larger percentage of cases in the young adult age group is concerning due to its potential in disrupting the work force in addition to social or familial consequences.

Serotype specific studies showed that the serotype responsible for the majority of the dengue cases in 2012 was DENV1. Previously in 2007, DENV3 was observed in the majority of cases in the Southern Province (Reller, 2012). DENV3 has also been thought to be the major cause behind severe epidemics in 1989 and 2000 when there was clade replacement for DENV3 genotype 3 from IIIA to IIIB (Messer et al., 2003; Kanakaratne, 2009). Until 2009, DENV2 and DENV3 were the predominant circulating DENV serotypes accounting for 86% of cases as opposed to the 7% by DENV1 (Sirisena et al., 2014). The most recent detection of DENV1 in Sri Lanka prior to this study was in 2009 where a new genotype for DENV1 was found through E-gene sequencing of Colombo samples (Tissera et al., 2011). Mapping of our results to previously published sequences

indicates that a majority of the DENV1 sequences belong to the DENV1 genotype 1 group (Tissera et al., 2011). We also found one case of DENV1 genotype 5, which is separate from the previously dominant DENV1 genotype 4 in Sri Lanka.

DENV4 sequence mapping showed that all of the DENV4 sequences belong to genotype 1, or the Southeast Asian genotype, which agrees with what has previously been found in the region (Kanakaratne, 2009). Overall, there is less literature available for DENV4 due to the limited sampling of that serotype. However, it has been shown to be the predominant serotype in DHF epidemics of 1986 and 1998 in Sri Lanka and should be monitored appropriately as studies show the possible association of DENV4 with DHF during secondary infection (Bennett et al., 2003; Mongkolsapaya et al., 2003; Deitz et al., 1996; Rigau et al., 1998).

For both DENV1 and DENV4 genotyped samples, clustering of Galle samples were observed for a small number of our samples. Such clustering may suggest a strain divergence from those found in Colombo and possible local transmission within Galle, but this requires further investigation.

4.2 Limitations

Findings of this study are limited to the southwestern region of Sri Lanka. Although this study is a one-site study, it was held at the largest tertiary care center in the southern province of Sri Lanka. In addition to the availability of free health care, Teaching

Hospital of Karapitiya (THK) sees a large number of patients that cover a wide geospatial spread, which can be seen even within the study patients through the self-reported distance to THK. Hence, we assume that a large representation of presentable diseases for Southern Sri Lanka can be observed in the patients that come to THK.

Laboratory limitations do exist within the study, particularly for serological testing. Cross-reactions can occur between dengue and other flaviviruses like West Nile virus, tick-borne encephalitis virus and yellow fever virus due to the limited specificity of antibodies. However, cross-reaction is not a major concern in this study since only Japanese encephalitis has been detected in the region our study encompasses (CDC, 2013). Additionally, our definitions for serological interpretation do not include all four of the proposed laboratory criteria set forth by the WHO. Adjusting our interpretations may increase the count for acute dengue; however, it should not drastically change the results presented here.

In addition to serological testing, limitations exist in following the molecular epidemiology of the dengue serotypes. We initially aimed to follow the geographic spread of dengue serotypes as dissemination of virus has been associated with urban to rural residencies. For DENV1, genotype 1 was first identified in Colombo during the 2009 – 2010 dengue season, which was also identified in our current study in Galle spanning the 2012-2013 dengue season. However, we are unable to ascertain when this was introduced to Galle due to the lack of samples between early 2008 and mid-2012.

Larger geographic coverage over similar temporal spread is necessary to conduct a more complete analysis of molecular epidemiology of DENV.

4.3 Implications for Future Research

In conclusion, we have aimed to describe the dengue season of 2012 through epidemiological and laboratory-based findings. Dengue serology conducted on a subset of paired sera samples indicated acute dengue was present in 64.83% of which 24.02 % were primary infections. Significant difference in the probability of acute dengue amongst children and adults agree with the notion that children are a more vulnerable population than adults. However, our results also show the mean age of patients to be 25 years, reflecting an age shift from a mean of 15 years that has been seen in various parts of Sri Lanka. Additionally, acute dengue cases were found to be different amongst urban to rural residences agreeing with the possibility that historically urban diseases are getting spread to more rural areas with advances in transportation of goods and people. Dengue seroprevalence in our study population was found to be 79.9%, which increased with age, as has been previously found in 2007 in our pilot study.

Specific dengue serotypes were confirmed through virus isolation and molecular testing indicating that amongst dengue positive samples 93.84% were DENV1 and 6.16% were DENV4. Serotype distribution for this dengue season differed from our study in 2007 where DENV2, DENV3 and DENV4 were identified with DENV3 causing a

majority of the dengue cases (Reller, 2012). Further phylogenetic analysis using full genome sequences of a representative subset of samples from 2012-2013 showed that the majority of DENV1 belonged to Asia genotype 1 and DENV4 belonged to Southeast Asia genotype 1. Our findings agree with what is found in the literature and specifically for DENV1 shows that genotype 1 strains initially identified in Colombo in 2009-2010 have now been identified in Galle in the 2012-2013 dengue season.

Results from this first year of surveillance hopefully will better inform the public and health officials on the state of dengue in the southern province of Sri Lanka. Additionally, it is important to monitor the evolving dynamic of different DENV serotypes that are causing cyclical epidemics to better understand the virus. Such information will hopefully benefit the scientific community's overall knowledge of the virus as movements towards vector control, treatments and vaccines are made. The continuation of surveillance programs is important in monitoring disease-causing agents. In order to move towards an active surveillance system, it is necessary to incorporate whole genome sequencing of representative samples across a geospatial and temporal spread to better understand virus dynamics and to detect introduction of evolving genotypes of viruses so that proper public health preparation can be made. Combined with whole genome sequencing, continued surveillance will likely remain costly. The importance of monitoring disease-causing agents, however, should outweigh

the costs as the knowledge will ultimately transform the current paradigm of retrospective study into one of active prediction and targeted, preventative action.

Appendix A

Table 5: PCR Cycling Parameters with Serotype-Fragment Specific Primer Pairs (* denotes primers previously published by Christenbury et al., 2010)

Fragment	PCR Primer Pair	Denaturing	Melting	Annealing	Amplification	Extension
DENV1 Fragment 1	D1-5F, D1-2084R D1-1F*, D1-2259R*	95°C, 2min 95°C, 2min	95°C, 2min 95°C, 2min	50°C, 20s 50°C, 20s	72°C, 1:30min 72°C, 1:30min	72°C, 3min 72°C, 3min
DENV1 Fragment 2	D1-854F, D1-3033R* D1-2201F*, D1-4561R D1-2065F, D1-4241R D1-3241F, D1-7537R D1-3735F, D1-7537R	95°C, 2min 95°C, 2min 95°C, 2min 95°C, 2min 95°C, 2min	95°C, 2min 95°C, 2min 95°C, 2min 95°C, 2min 95°C, 2min	47°C, 20s 47°C, 20s 47°C, 20s 47°C, 20s 47°C, 20s	72°C, 1:30min 72°C, 1:30min 72°C, 1:30min 72°C, 1:30min 72°C, 1:30min	72°C, 3min 72°C, 3min 72°C, 3min 72°C, 3min 72°C, 3min
DENV1 Fragment 3	D1-4221F, D1-6461R D1-4213F*, D1-6537R* D1-4541F, D1-7537R D1-5243F, D1-7537R	95°C, 2min 95°C, 2min 95°C, 2min 95°C, 2min	95°C, 2min 95°C, 2min 95°C, 2min 95°C, 2min	50°C, 20s 50°C, 20s 50°C, 20s 50°C, 20s	72°C, 1:30min 72°C, 1:30min 72°C, 1:30min 72°C, 1:30min	72°C, 3min 72°C, 3min 72°C, 3min 72°C, 3min
DENV1 Fragment 4	D1-6442F, D1-8519R D1-6216F*, D1-8577R* D1-7921F, D1-9613R	95°C, 2min 95°C, 2min 95°C, 2min	95°C, 2min 95°C, 2min 95°C, 2min	50°C, 20s 50°C, 20s 50°C, 20s	72°C, 1:30min 72°C, 1:30min 72°C, 1:30min	72°C, 3min 72°C, 3min 72°C, 3min
DENV1	D1-8540F, D1-10693R D1-8558F, D1-10693R D1-8211F*, D1-	95°C, 2min 95°C, 2min 95°C, 2min	95°C, 2min 95°C, 2min 95°C,	50°C, 20s 50°C, 20s 50°C, 20s	72°C, 1:30min 72°C, 1:30min 72°C,	72°C, 3min 72°C, 3min 72°C,

	10450R*		2min		1:30min	3min
	D1-8994F, D1-10693R	95°C, 2min	95°C, 2min	50°C, 20s	72°C, 1:30min	72°C, 3min
	D1-9620F, D1-10693R	95°C, 2min	95°C, 2min	50°C, 20s	72°C, 1:30min	72°C, 3min
DENV4 Fragment 1	D4-1F*, D4-2579R*	95°C, 2min	95°C, 2min	50°C, 20s	72°C, 1:30min	72°C, 3min
	D4-9F, D4-2107R	95°C, 2min	95°C, 2min	50°C, 20s	72°C, 1:30min	72°C, 3min
DENV4 Fragment 2	D4-2065F, D4-4246R	95°C, 2min	95°C, 2min	50°C, 20s	72°C, 1:30min	72°C, 3min
	D4-1953F*, D4-4926R*	95°C, 2min	95°C, 2min	50°C, 20s	72°C, 1:30min	72°C, 3min
	D4-2968F, D4-4246R	95°C, 2min	95°C, 2min	50°C, 20s	72°C, 1:30min	72°C, 3min
	D4-2968F, D4-6463R	95°C, 2min	95°C, 2min	50°C, 20s	72°C, 1:30min	72°C, 3min
	D4-3836F, D4-6163R	95°C, 2min	95°C, 2min	50°C, 20s	72°C, 1:30min	72°C, 3min
	D4-3836F, D4-7349R	95°C, 2min	95°C, 2min	50°C, 20s	72°C, 1:30min	72°C, 3min
DENV4 Fragment 3	D4-4226F, D4-6463R	95°C, 2min	95°C, 2min	50°C, 20s	72°C, 1:30min	72°C, 3min
	D4-4814F*, D4-7330R*	95°C, 2min	95°C, 2min	50°C, 20s	72°C, 1:30min	72°C, 3min
	D4-5243F, D4-7537R	95°C, 2min	95°C, 2min	50°C, 20s	72°C, 1:30min	72°C, 3min
DENV4 Fragment 4	D4-6444F*, D4-8531R*	95°C, 2min	95°C, 2min	50°C, 20s	72°C, 1:30min	72°C, 3min
	D4-7224F, D4-9708R	95°C, 2min	95°C, 2min	50°C, 20s	72°C, 1:30min	72°C, 3min
DENV4 Fragment 5	D4-7911F, D4-10626R	95°C, 2min	95°C, 2min	50°C, 20s	72°C, 1:30min	72°C, 3min
	D4-8512F, D4-10626R	95°C, 2min	95°C, 2min	50°C, 20s	72°C, 1:30min	72°C, 3min
	D4-9269F*, D4-10643R*	95°C, 2min	95°C, 2min	50°C, 20s	72°C, 1:30min	72°C, 3min

Appendix B

Table 6: List of Sequencing Primers Used for Full Genome Sequence (*denotes primers that were previously published by Christenbury et al., 2010. All other primers are unpublished primers or were designed specifically for this study)

Serotype	Primer	Sequence
DENV-1	d1a4*	5'-CACTCCACTGAGTGAATTCTCTCT-3'
DENV-1	d1a8*	5′-CAAGTCCCATCAATATAGCTGC-3′
DENV-1	d1a12*	5′-CCTCGTCCTCAATCTCTGGTAG-3′
DENV-1	d1a17*	5′-CCAATGGCYGCTGAYAGTCT-3′
DENV-1	d1a5B*	5'-*TTTGTCGGTCTGGGGGGGTAT*AGAACCTGTTGATTCAACRGC-3'
DENV-1	d1s1C*	5'-*GATGAGGGAAGATGGGG*AGTTGTTAGTCTACGTGGAC-3'
DENV-1	d1s6*	5′-GGYTCTATAGGAGGRGTGTTCAC-3′
DENV-1	d1s8*	5′-ACAAACAGCAGGGCCRTGGCA-3′
DENV-1	d1s14*	5′-ATGGRGAAAGGAACAACCAG-3′
DENV-1	d1s18*	5′-CCACYCATGAAATGTAYTGGGT-3′
DENV-1	d1a21*	5'-CATYGCAATRAGRGTGCACAT-3'
DENV-1	d1s3*	5'-AAACGTTCCGTSGCACTGGC-3'
DENV-1	d1a20*	5'-CGTCTTCAAGAGTTCAATGTCC-3'
DENV-1	d1s4*	5'-TGTGTGTCGMCGAACGTT-3'
DENV-1	d1a19*	5'-GTTTGTGGACRAGCCATGATT-3'
DENV-1	d1s5*	5'-GCAATGCACACYGCGTTG-3'
DENV-1	d1a18*	5'-AAAGGTGGYTCYGYYTCAAT-3'
DENV-1	d1a17*	5'-CCAATGGCYGCTGAYAGTCT-3'
DENV-1	d1s6*	5'-GGYTCTATAGGAGGRGTGTTCAC-3'
DENV-1	d1s7*	5'-GGCCCAAGGRAARAAAATG-3'
DENV-1	d1a16*	5'-CARCTTCCARGTYTCGTTCTT-3'
DENV-1	d1a15*	5'-GCATYTTTCTRCTCCATCTGGATC-3'
DENV-1	d1s9*	5'-CCTAGCYYTGATGGCYACTTT-3'
DENV-1	d1a14*	5'-CCGGAAGCCATGTTGTTTT-3'
DENV-1	d1s10*	5'-RGCYGGSCCACTAATAGCT-3'
DENV-1	d1a13*	5'-TTCCACTTCYGGAGGGCT-3'
DENV-1	d1s10*	5'-RGCYGGSCCACTAATAGCT-3'
DENV-1	d1a13*	5'-TTCCACTTCYGGAGGGCT-3'
DENV-1	d1s11*	5'-AAGAGRCTGGAACCRAGYTGGGC-3'

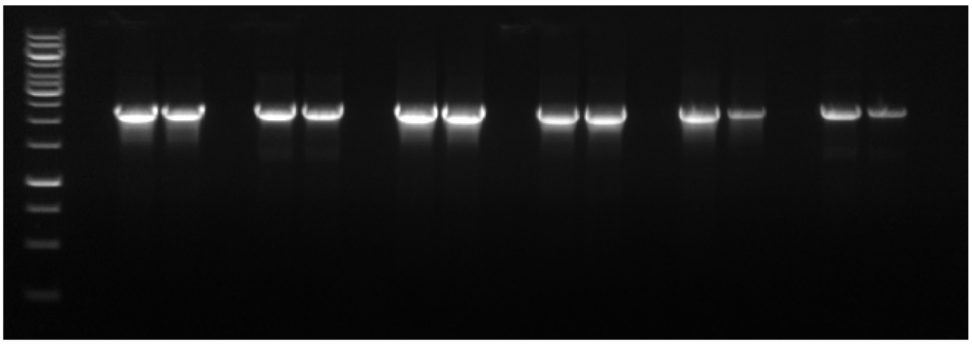
DENV-1	d1s12*	5'-AAATGGCAGAGGCGCTCAAGGG-3'
DENV-1	d1a11*	5'-CRTAGCCTGARTTCCATGATCT-3'
DENV-1	d1s13*	5'-ACAAAAAAYAAYGACTGGGACTAT-3'
DENV-1	d1a10*	5'-TCTCTCYGGCTCAAAGAGGG-3'
DENV-1	d1a9*	5'-CCAGTYARCACAGCTATCAAAGC-3'
DENV-1	d1s14*	5'-ATGGRGAAAGGAACAACCAG-3'
DENV-1	d1a9*	5'-CCAGTYARCACAGCTATCAAAGC-3'
DENV-1	d1s15*	5'-GGATAGCGGCCTCYATCATACT-3'
DENV-1	d1s16*	5'-GCAAARGCYACTAGAGAAGCTCAA-3'
DENV-1	d1a7*	5'-CCTACCTCCTCCTARAGATTTCA-3'
DENV-1	d1s17*	5'-GAAACRACYAAACAYGCAGTG-3'
DENV-1	d1a6*	5'-AGRACACGTAACGTTCTWCCTTC-3'
DENV-1	d1a5*	5'-GGRATRACATCCCATGGTTT-3'
DENV-1	d1s18*	5'-CCACYCATGAAATGTAYTGGGT-3'
DENV-1	d1s19*	5'-GCCARGTGGTTATGGGGTTT-3'
DENV-1	d1s20*	5'-GGATGATCTTCAGAATGAGGC-3'
DENV-1	d1a3*	5'-YACRCARTCATCTCCRCTGAT-3'
DENV-1	d1s21*	5'-TYATGAAGGATGGGAGGGA-3'
DENV-1	d1a2*	5'-DTCTTCCCAACTGGAYACATG-3'
DENV-1	d1s1*	5'-TRGCTCCATCGTGGGGAT-3'
DENV-1	D1-3F	5'-GGCTATCCTACAAAGTTGCCTCAG-3'
DENV-1	D1-3927F	5'-GGCTACCTTGCTGTCC-3'
DENV-1	D1-5986F	5'-GGACAGAAGCAAAAATGCTCC-3'
DENV-1	D1-8282F	5'-CCAGAATGTTGCTAAATCG-3'
DENV-1	D1-6645R	5'-GCCATCACGCAGAGTAGG-3'
DENV-1	D1-7674R	5'-CCCACTCCTTTTATAGG-3'
DENV-4	d4a3*	5’-TGTGRAARTGGTGGGAGCAAAA-3’
DENV-4	d4a8*	5’-ACYTGCCCTAATTGCTTTTCAAA-3’
DENV-4	d4a14*	5’-TTGGTRAACYACTCCATTTCC-3’
DENV-4	d4a18*	5’-GGGCATTYAATATTGCAGACGCTA-3’
DENV-4	d4a5B*	5’-*TTTGTCGGTCTGGGGGGGTAT*AGAACCTGTTGGATCAACAAC-3'
DENV-4	d4s1C*	*5′-GATGAGGGAAGATGGGG*AGTTGTTAGTCTGTGTGGACCGAC-3'
DENV-4	d4s5*	5’-CTCCGTGTAAAGTCCCCATAGAGA-3’

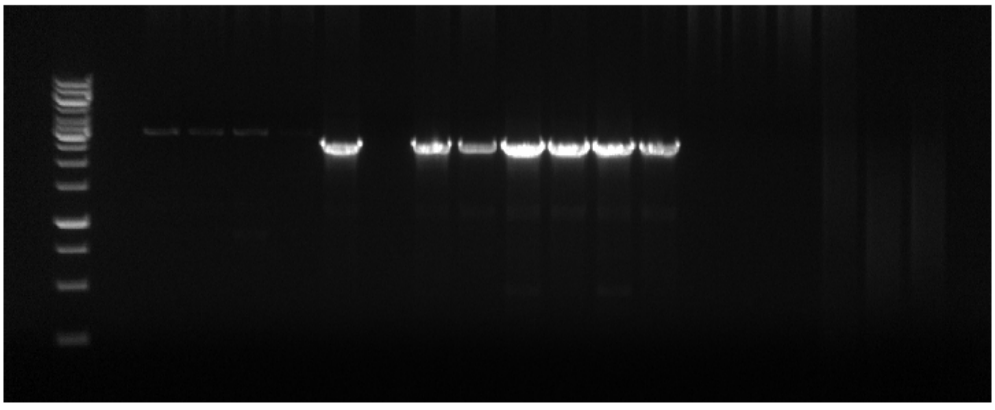
DENV-4	d4s11*	5’-CCTMGCYATAGAACCAGGAAAAAATC-3’
DENV-4	d4s16*	5’-AGRACAGCTGCTGGGATCATGA-3’
DENV-4	d4s21*	5’-GAAAGACATYCCGCAGTGGGAA-3’
DENV-4	d4s2*	5’-AACAAATGCACYCTYATTGCCA-3’
DENV-4	d4s3*	5’-TTTGAACTGACYAAGACAACAGC-3’
DENV-4	d4s4*	5’-TGGACAGCAGGAGCAGACACAT-3’
DENV-4	d4s6*	5’-GYTCCATTGGCAAGATGTTTGAG-3’
DENV-4	d4a2*	5’-AACYTGGTGYACAGCCTTTCCC-3’
DENV-4	d4a4*	5’-CATGCTGTGTTTCTGCCATCTC-3’
DENV-4	d4a6*	5’-TGGGTGTCTCCATTGTGGACTG-3’
DENV-4	d4a7*	5’-TACATGACCCAGGTRGACGTGAG-3’
DENV-4	d4s7*	5’-GTTGTGTGGYGTCATGGARTGG-3’
DENV-4	d4s8*	5’-CATATGGATGAAATTCCGAGAAGGA-3’
DENV-4	d4s9*	5’-GGAGATTAGGCCCTTGAGTGAAAA-3’
DENV-4	d4s10*	5’-CATTRGTCATGGCTTGGAGGACC-3’
DENV-4	d4s12*	5’-GAAACTGGCRCTGATAACAGTRTCA-3’
DENV-4	d4a9*	5’-TTTCCCRAAYAACCCTCTTTGC-3’
DENV-4	d4a10*	5’-ACCAAACCCACAGCCATTATGC-3’
DENV-4	d4a11*	5’-GAGRCCTCCYAGGATGATAGCAC-3’
DENV-4	d4a12*	5’-ATGAGCATCTGRCTTTCCAGCAC-3’
DENV-4	d4a13*	5’-TCYTGTTDATGATRGGAGARCCAGA-3’
DENV-4	d4a15*	5’-TATTGCAGACGCTAGYCTCGCTG-3’
DENV-4	d4s13*	5’-CTTSTAGTGTCGCRGCTAGAGGATA-3’
DENV-4	d4s14*	5’-TGCYCACTGGACAGAAGCAAAGAT-3’
DENV-4	d4s15*	5’-TAGGTGCTATGACAGCMGGYATCT-3’
DENV-4	d4s17*	5’-ACCTTGACAGCATCCYTAGTCATG-3’
DENV-4	d4a16*	5’-TCAATTGATTGTCTTGTGGGGTC-3’
DENV-4	d4a17*	5’-CTTTCCCCTGTGAAGCACCAT-3’
DENV-4	d4a19*	5’-TCAACTGGATHACTYTCTTTCCCG-3’
DENV-4	d4a20*	5’-TTGTCTTTCCRGCTCCGGGGT-3’
DENV-4	d4s18*	5’-GGTCBTATTACATGGCGACACTCA-3’
DENV-4	d4s19*	5’-GCACAAAGAAACCTGGCAYTATGA-3’
DENV-4	d4s20*	5’-GGAATTTGAAGCCCTGGGTTTT-3’
DENV-4	d4s22*	5’-AAAGACATYCCGCAGTGGGA-3’
DENV-4	d4a21*	5’-CATCTGTTCYGTGATCAGTTCYTCA-3’
DENV-4	d4a22*	5’-ARCCAATTGGCTGTCGTGGT-3’
DENV-4	d4a23*	5’-GARTTCCTGGAYARCGGGCA-3’
DENV-4	d4a24*	5’-CCATCTYTYAGGGCAGACTTGG-3’
DENV-4	d4s23*	5’-GACAAGACTCCAGTCCATTCGTG-3’
DENV-4	d4a25*	5’-TTCCGATCAGRTTCCTGACCTG-3’
DENV-4	D4F1-28F	5'-GTCTGTGTGGACCGACAAGCACAG-3'

DENV-4	D4F1-65F	5'-GCTTGCTTAACACAGTTCTAACAG-3'
DENV-4	D4-2387F	5'-CCTCAATGGCAATGTCATGC-3'
DENV-4	D4-3836F	5'-CCATGACAACGGTGCTTTC-3'
DENV-4	D4-6002F	5'-GATGAAGATCATGCCCACTG-3'
DENV-4	D4-3532R	5'-GTTTCTGATGTACCCTGTCC-3'
DENV-4	D4-6163R	5'-CTCTCCATCAATGGCTTGG-3'
DENV-4	D4-8267R	5'-CCATGGCTCCACCATCTTC-3'
DENV-4	D4-9514R	5'-GGTTATGCATGTCATCTCG-3'

Appendix C

Representative images of PCR product separated using 1% agarose TAE gel and visualized under UV light after staining with ethidium bromide. 1kb ladders were used for each gel to locate desired bands.





Appendix D

From August through October of 2013, I engaged in a capacity building project at the University of Ruhuna in Galle, Sri Lanka as a member of the Duke-Ruhuna Febrile Illness Study Team. My primary objective was to establish two in-house ELISA assays that were being used at Duke-NUS Medical School in Singapore with proper verification methods. My secondary objective was to train selected in-country personnel on the two ELISA assays.

Prior to the project in Galle, I spent three months at Duke-NUS Medical School in Singapore predominantly running the two ELISA assays for dengue serology – IgM and IgG assays. Through the hundreds of assays, I was able to not only demonstrate consistency in my experiments but to gain a better understanding of the assay. Once arriving at the University of Ruhuna, I initially began with a trial ELISA with a commercial kit to get accustomed to the set-up at the laboratory. I then started testing with the IgG ELISA followed by the IgM ELISA making necessary adjustments to the protocols due to resource limitations. Notable adjustments included substituting manual washing to all wash steps due to the lack of an automatic washer, the creation of in-house controls for both assays and the usage of a full 96-well plate in lieu of the inner 60-wells for the IgM assay.

After the establishment of the two assays, selected samples from the runs I had completed in Singapore were selected to understand the accuracy of the ELISA assays at

the University of Ruhuna. This was done in efforts to minimize the number of variables to the environment and necessary protocol adjustments. Once results were replicated, these same sera were used for training purposes.

A total of five in-country individuals were trained in both the IgG and IgM assay. After the initial group training, training was switched to an individual "one-on-one" basis to limit conversation to English only – due to my lack of Sinhala knowledge beyond basic conversational phrases. Given this time constraint, all five individuals were given the opportunity to run one IgG and one IgM ELISA test with the exception of one technician who went on for further training. This lab technician was chosen under the discretion of my supervisor after discussing preliminary results, availability in terms of other laboratory work and flexibility with time. This individual completed two additional IgM tests where results were replicated with 100% accuracy both times.

Aside from the training, I was also asked to test 50 samples to which I was blinded in terms of results – these samples were chosen from the most recent shipment to Singapore, which arrived in Singapore after I had left. A total of 60 samples were tested on the IgM assay after being selected for volume availability and confirmation of sera availability in Singapore. A different aliquot of the same samples were tested by a different researcher in Singapore and upon comparison, results obtained in Galle were confirmed with those in Singapore.

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